

Figure 1 A

SEQ ID NO: 1

/translation="MGSVLSTDSGKSAPASATARALERRRDPELPVTSFDCAVCLEVL

HQPVRTRCGHVFCRSCIATSLKNNKWTCPYCRA YLPSEGVPATDVAKRMKSEY
KNCAE

CDTLVCLSEMRAHIRTCQKYIDKYGPLQELEETAARCVCPFCQRELYEDSLLDHC
ITH

HRSERRPVFCPLCRLIPDENPSSFSGNLIRHLQVSHTLFYDDFIDFNIIIEALIRRVL
DRSLLEYVNHSNTT"

Figure 1 B

SEQ ID NO: 2

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SEQ ID NO: 3

Figure C

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SEQ ID NO: 4

Figure 1 D

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Figure 1

TRAC1 genomic region:

SEQ ID NO: 5

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Figure cont'd

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SEQ NO: 5
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Figure 1 E cont'd

SEQ NO:5 cont'd

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Figure 1 F

SEQ ID NO: 6

Mouse TRAC1 cDNA sequence:

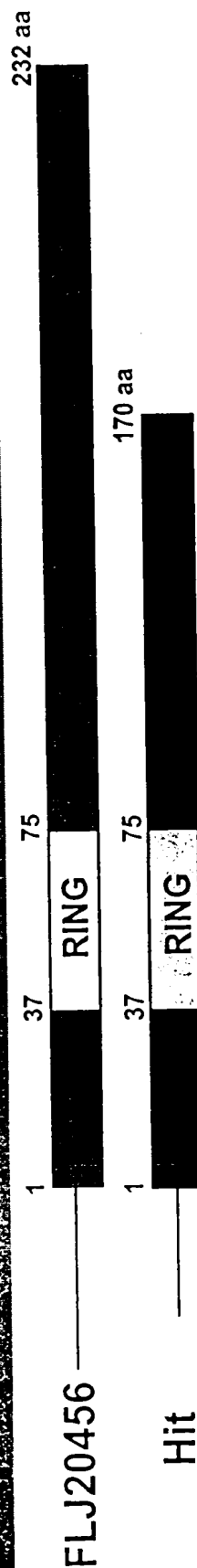
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Mouse TRAC1 protein (3rd frame)

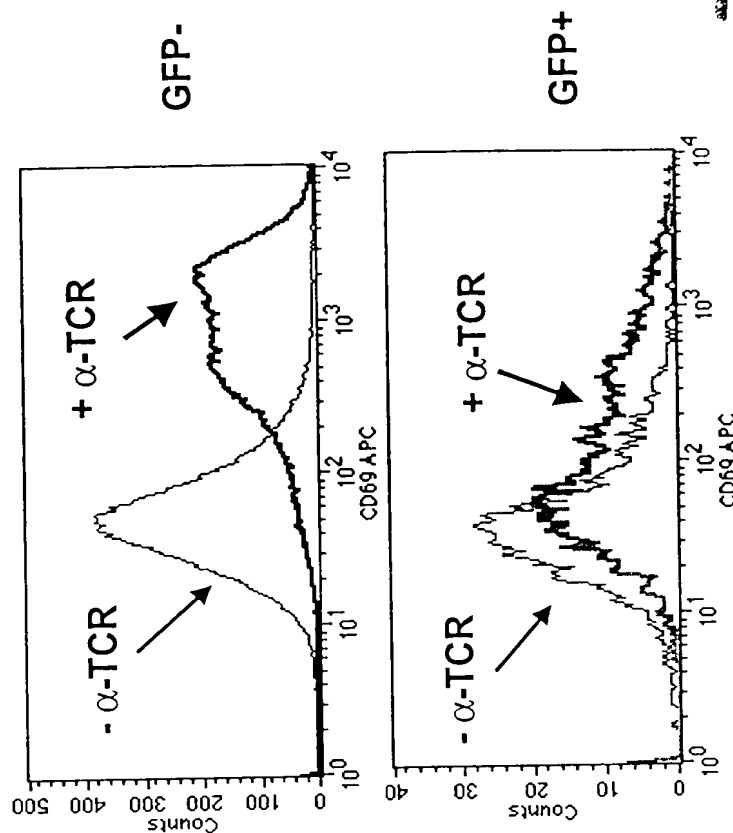
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FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells



Phenotype Transfer



Original clone

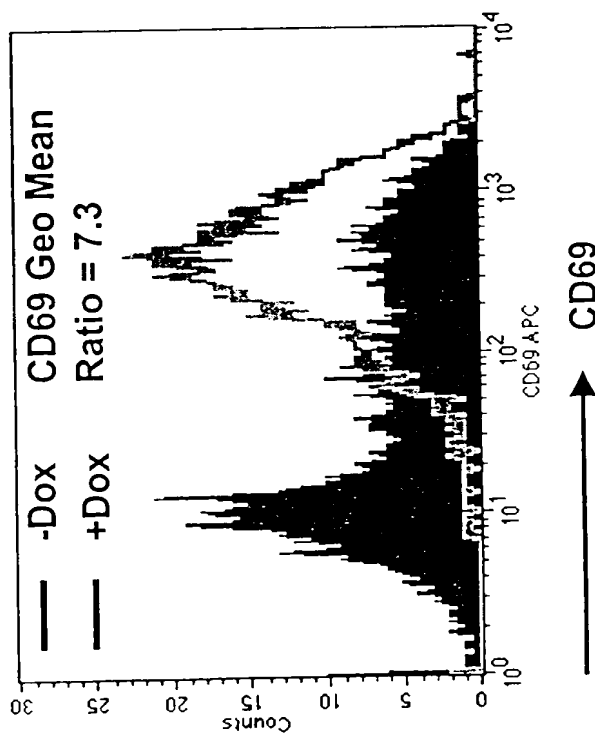


Figure 2

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

232 aa

75

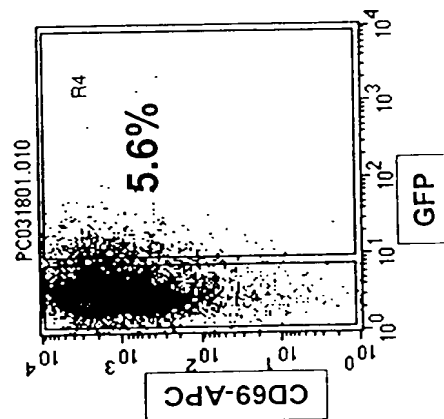
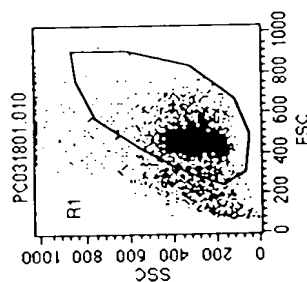
37

FLJ20456

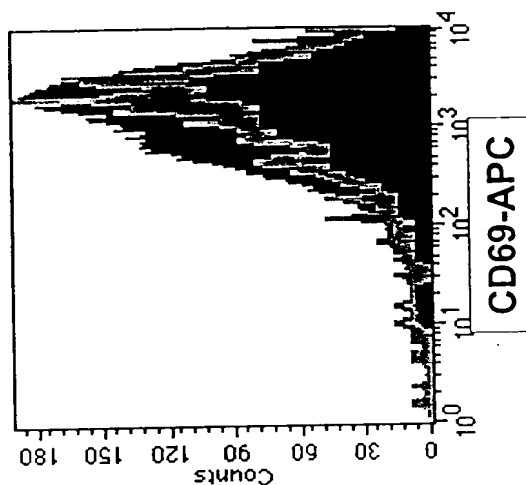
RING

- Pfu PCR product amplified from a capped human brain cDNA library.
- One N to S polymorphism with FLJ20456 NM_017831.1 at amino acid 186, present in EST database.

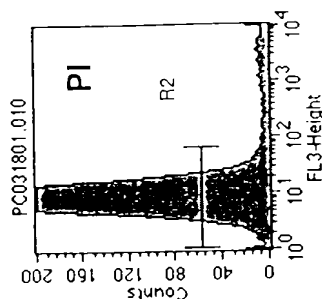
JurkatN 32H



300ng/ml C305



GFP- 1070.5
GFP+ 1219.9
Ratio = 0.88



Figure

Hit FLJ20456 Hit Specifically Inhibited T Cell Activation but not B Cell Activation

232 aa

FLJ20456

RING

37

75

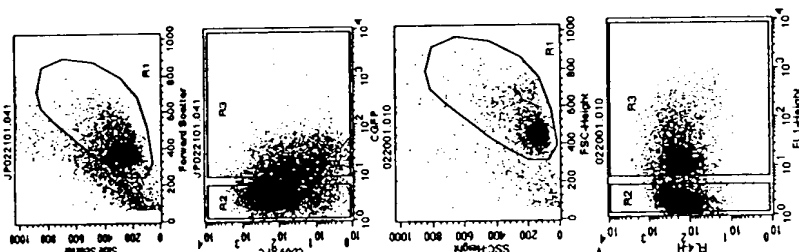
Hit

RING

37

75

170 aa



Stimulation:
300ng/ml C305

GFP- 141.2
GFP+ 28.8
Ratio = 4.9

Stimulation:
300ng/ml α -IgM

GFP- 183.1
GFP+ 183.3
Ratio = 1.0

Infect Jurkat
and
Assay

Infect BJAB
and
Assay

Transfect
Phoenix cells with
TIG-FLJ20456 hit



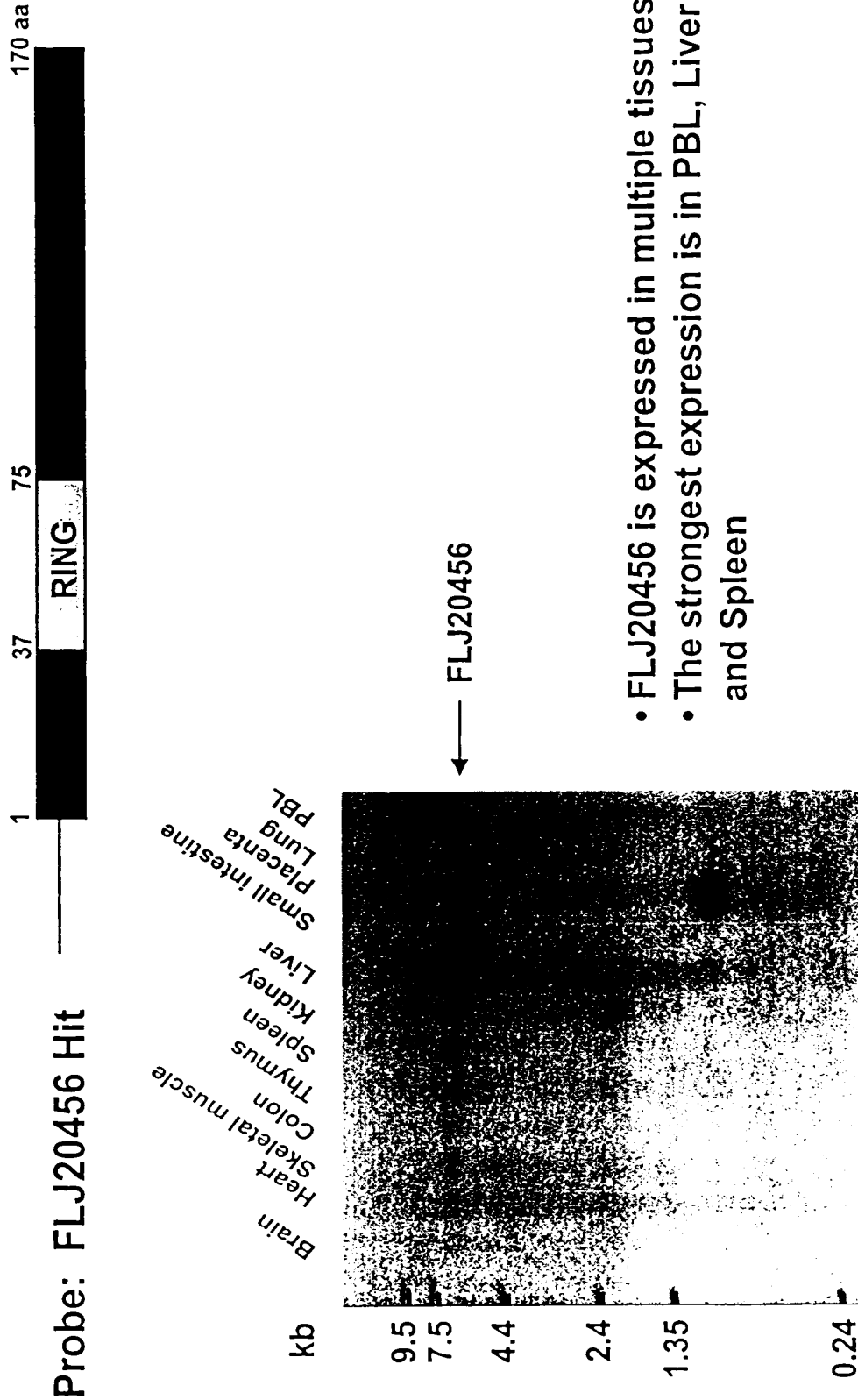
Viral supernatant

CD69-APC

CD69-APC

Figure 4

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs



- FLJ20456 is expressed in multiple tissues
- The strongest expression is in PBL, Liver and Spleen

Figure 6

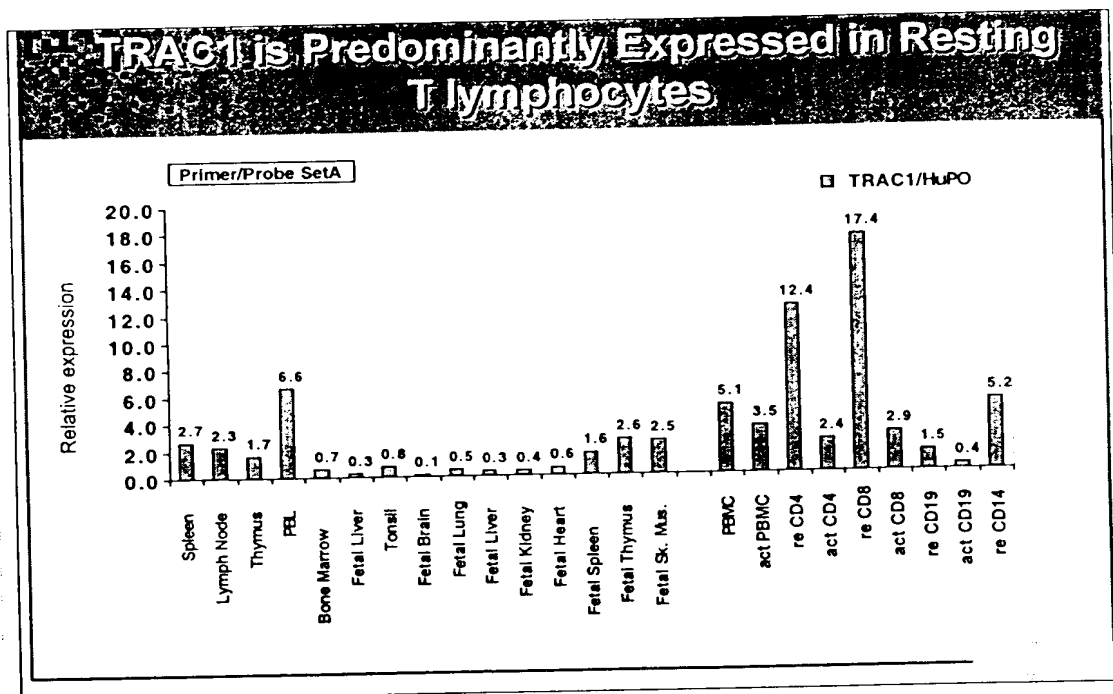


Figure 7

[illegible][illegible][illegible]

| | | | | | | | | | | | | | | | | | |
|--------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|
| Consensus #1 | V | C | P | C | P | P | P | P | P | N | H | Y | F | E | E | S | |
| FLJ20456.pep | P | V | F | C | P | L | C | R | L | I | P | D | E | N | P | S | 233 |
| zif313.pep | S | V | F | C | P | I | C | A | S | M | P | W | G | D | P | N | 229 |
| STRIN.pep | P | V | T | C | P | I | C | V | S | L | P | W | G | D | P | S | 246 |

| Percent Identity | | | | |
|------------------|----------|----------|----------|---|
| | 1 | 2 | 3 | |
| 1 | ████████ | 26.6 | 22.3 | 1 |
| 2 | 130.4 | ████████ | 27.9 | 2 |
| 3 | 140.9 | 134.7 | ████████ | 3 |

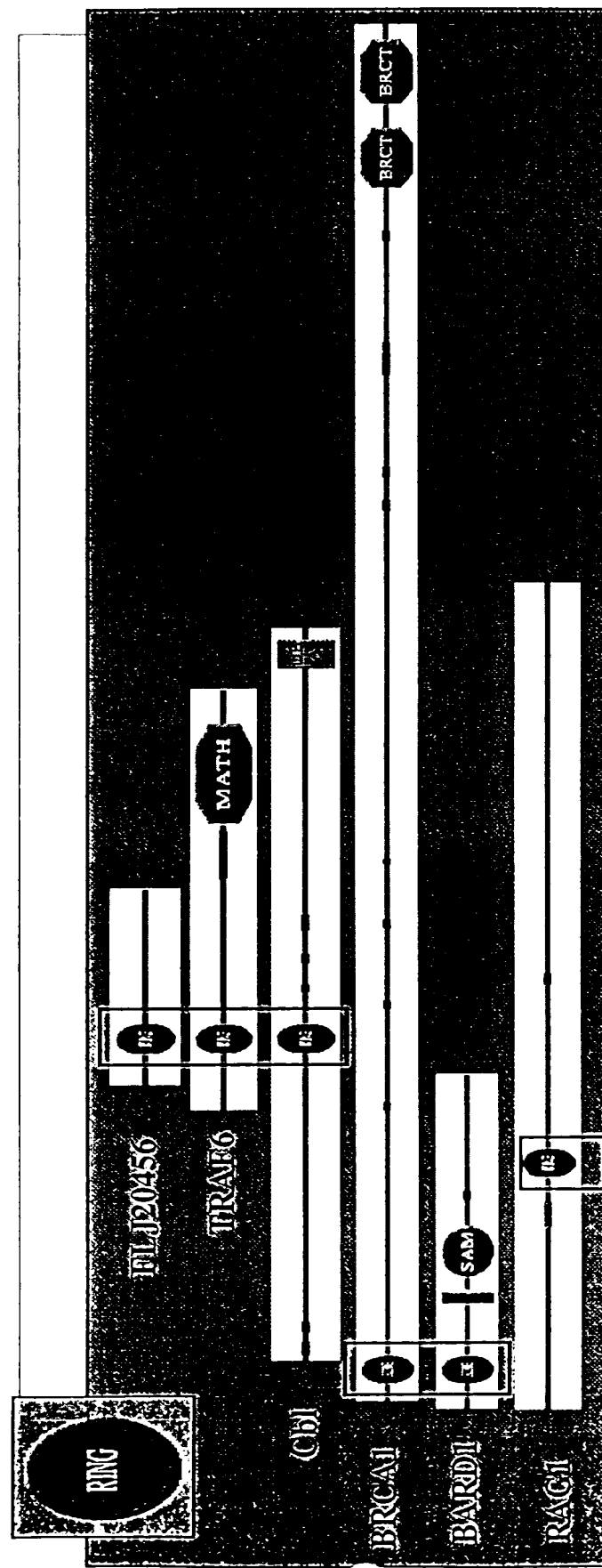
divergence

- All three sequences are human
- Murine sequences are not shown

Alignment of RING Domain Sequences from Various Human Proteins

Consensus #1

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|----|----|
| FLJ20456.Ring | V | T | S | F | D | C | A | V | C | L | E | V | L | H | Q | P | V | R | T | R | - | C | G | H | V | F | C | R | S | C | I | A | T | S | L | - | - | K | N | N | K | W | T | C | B | Y | C | R | A | Y | L | P | - | S | 50 | |
| znf313.Ring | L | G | R | F | T | C | P | V | C | L | E | V | Y | E | K | P | V | Q | V | P | - | C | G | H | V | F | C | S | A | C | L | Q | E | C | L | - | - | K | P | K | P | V | C | G | V | C | R | S | A | L | A | - | P | 50 | | |
| STRIN.Ring | E | D | D | F | Y | C | P | V | C | Q | E | V | L | K | T | P | V | R | T | A | - | C | Q | H | V | F | C | R | K | C | F | L | T | A | M | - | - | R | E | S | G | A | H | C | F | L | C | B | G | N | V | - | T | 50 | | |
| TRAF6.Ring | E | S | K | Y | E | C | P | I | C | L | M | A | L | R | E | A | V | Q | T | P | - | C | G | H | R | E | F | C | K | A | C | I | I | K | S | I | - | - | R | E | D | A | G | H | K | C | P | V | - | D | N | E | I | L | E | 50 |
| c-Col.Ring | S | T | F | Q | L | C | I | C | I | C | A | E | L | I | K | E | P | V | I | E | P | - | C | G | H | L | M | C | T | S | C | L | T | S | W | - | - | Q | E | S | E | G | Q | Q | C | P | F | C | R | C | E | I | - | K | G | 50 |
| BRCA1.Ring | - | - | - | L | E | C | P | I | C | L | E | L | I | K | E | P | V | S | T | K | - | C | L | I | F | C | R | K | F | C | M | L | T | L | N | Q | K | K | G | P | S | Q | C | P | L | C | K | N | E | I | T | K | R | 50 | | |
| BAR.Ring | V | S | E | F | S | C | H | C | Y | D | I | L | V | N | P | T | - | T | L | N | - | C | G | H | S | F | C | R | H | C | L | A | L | W | W | A | - | S | S | K | K | T | E | C | P | E | C | R | E | K | - | - | W | E | 50 | |
| RAG1.Ring | V | K | S | I | S | C | Q | I | C | E | H | I | L | A | D | P | V | E | T | N | - | C | K | H | V | F | C | R | V | C | I | L | R | C | L | - | - | K | V | M | G | S | Y | C | B | S | C | R | Y | P | C | F | - | P | 50 | |

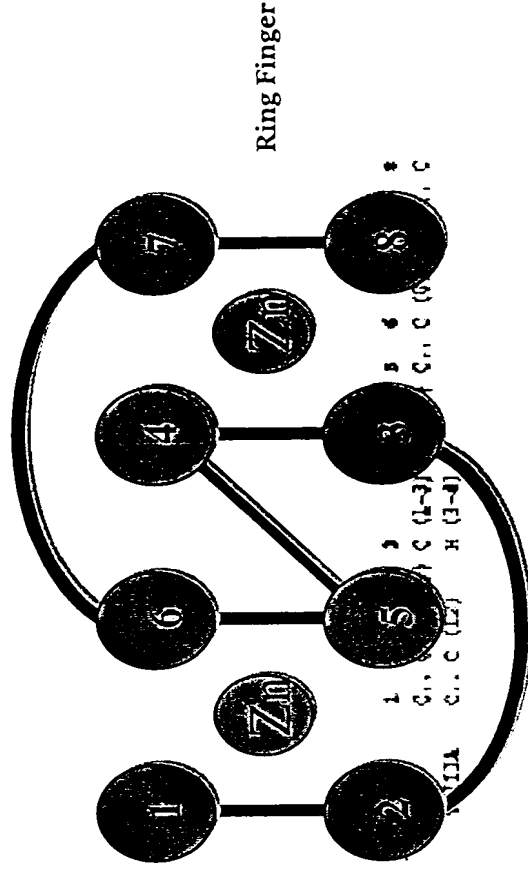


Figures

RING finger vs. Zinc finger proteins

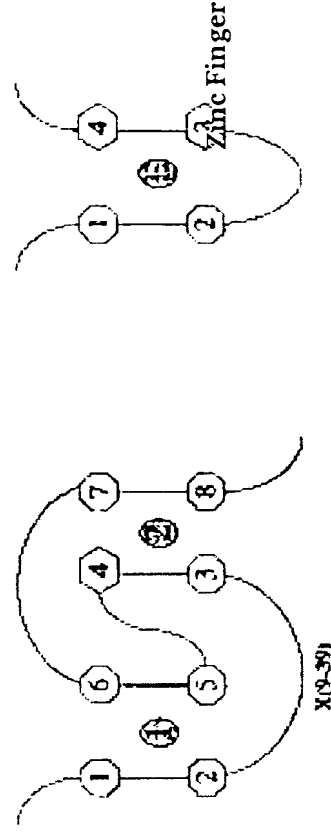
Ring-HC: C_3HC_4 = Cys in position 5
 Ring H2: $C_3H_2C_3$ = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that coordinate two zinc atoms to form a cross-brace structure



B.

C.



- Ring fingers are structurally distinct from zinc fingers

Figure 9

Ubiquitin Pathway Components

- E1: ubiquitin-activating enzyme, with a major isoform that may work broadly
- E2: ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3
- E3: ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins
- Proteasome-a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.

Enzymology of Ubiquitynation

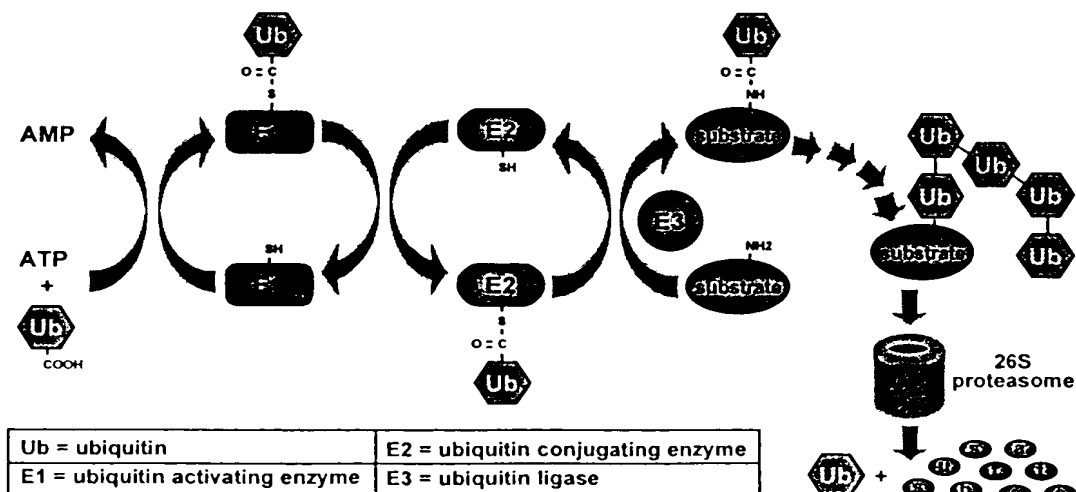
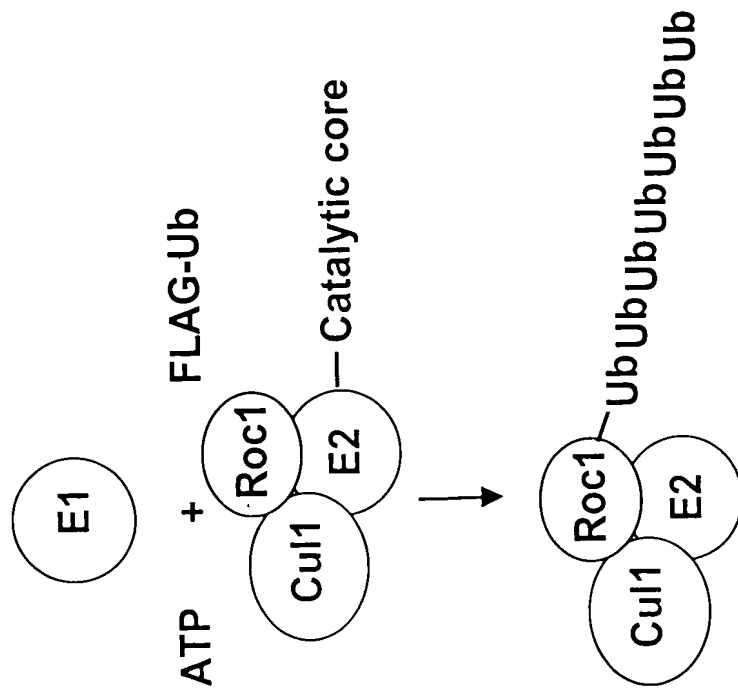


Figure 11a

A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis



The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Reaction Components

E1:

E2 (UbcH5): GST-fusion (cleaved), *E. coli*

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus

Ubiquitin: FLAG-tagged, *E. coli*



Figure 11 B

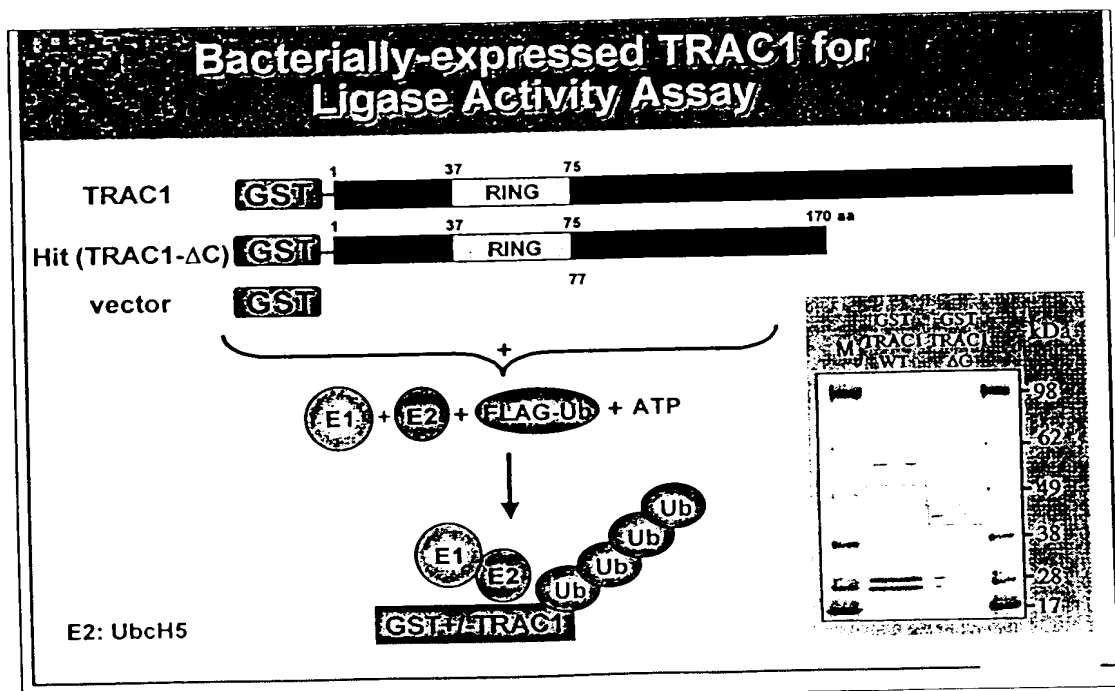
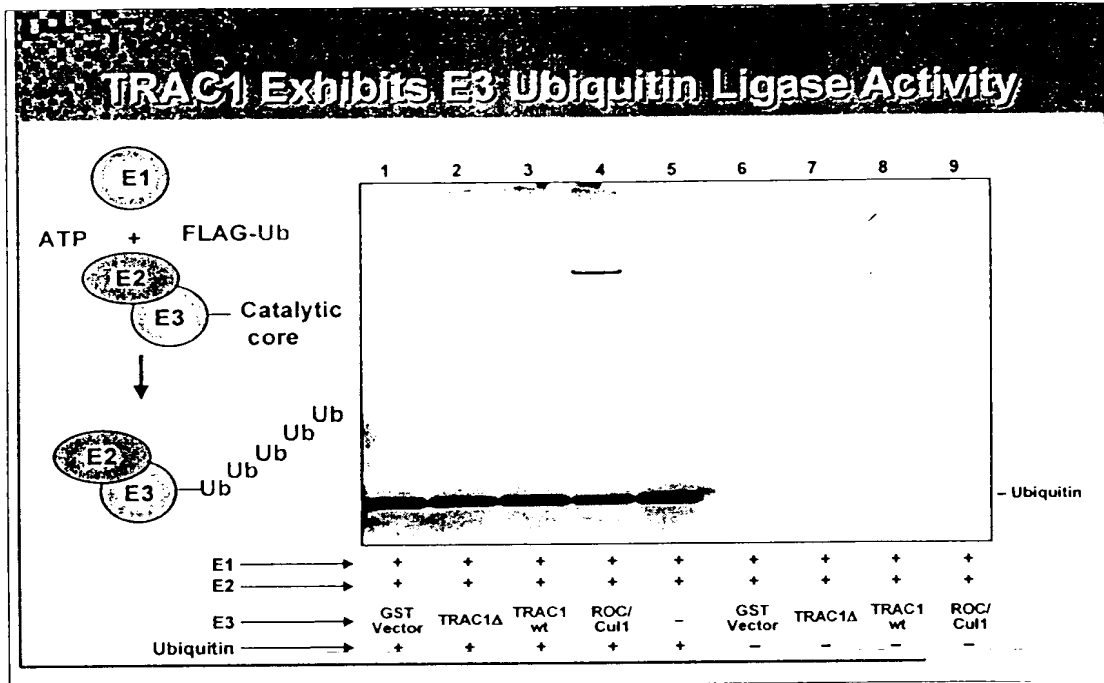


Figure 12

12A



12B

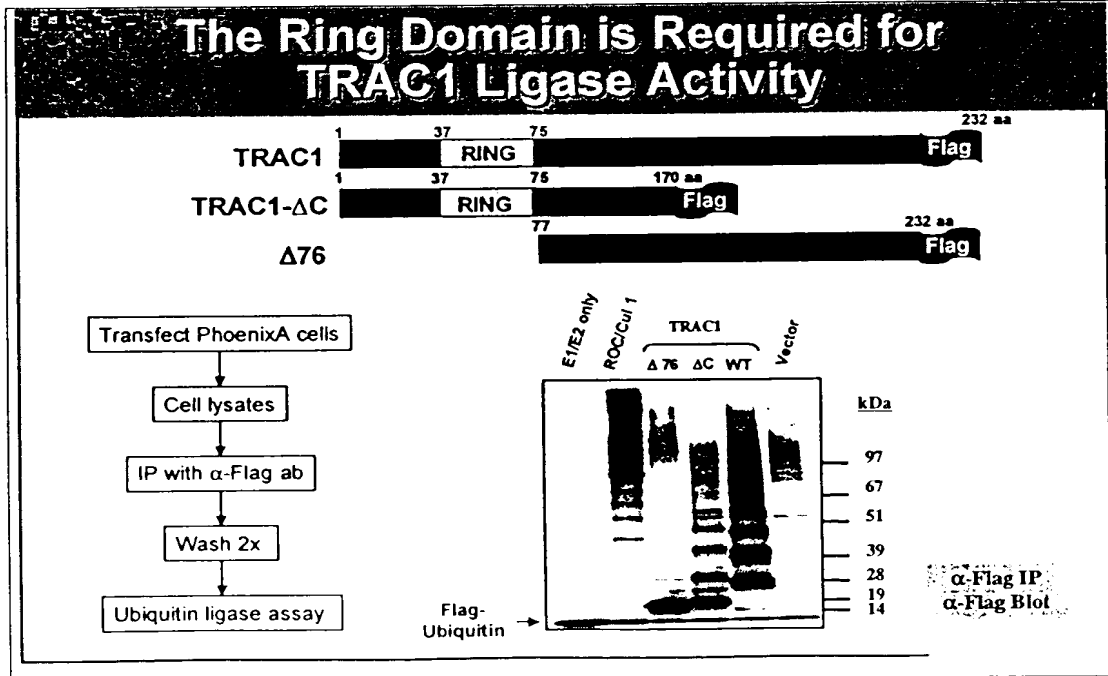


Figure 13

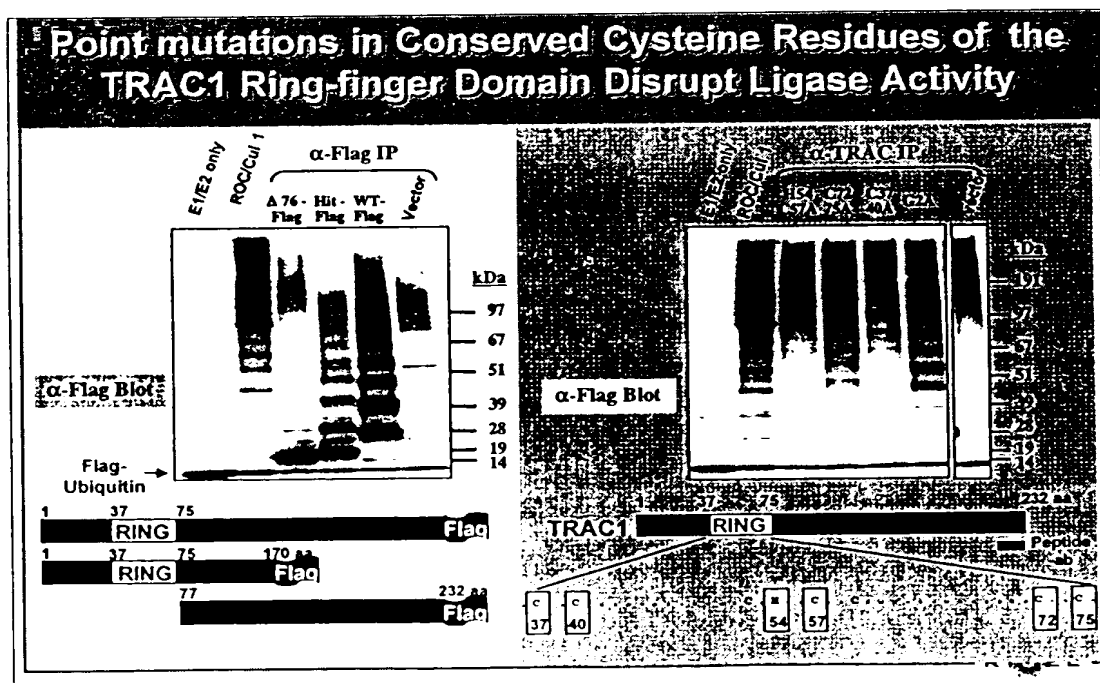
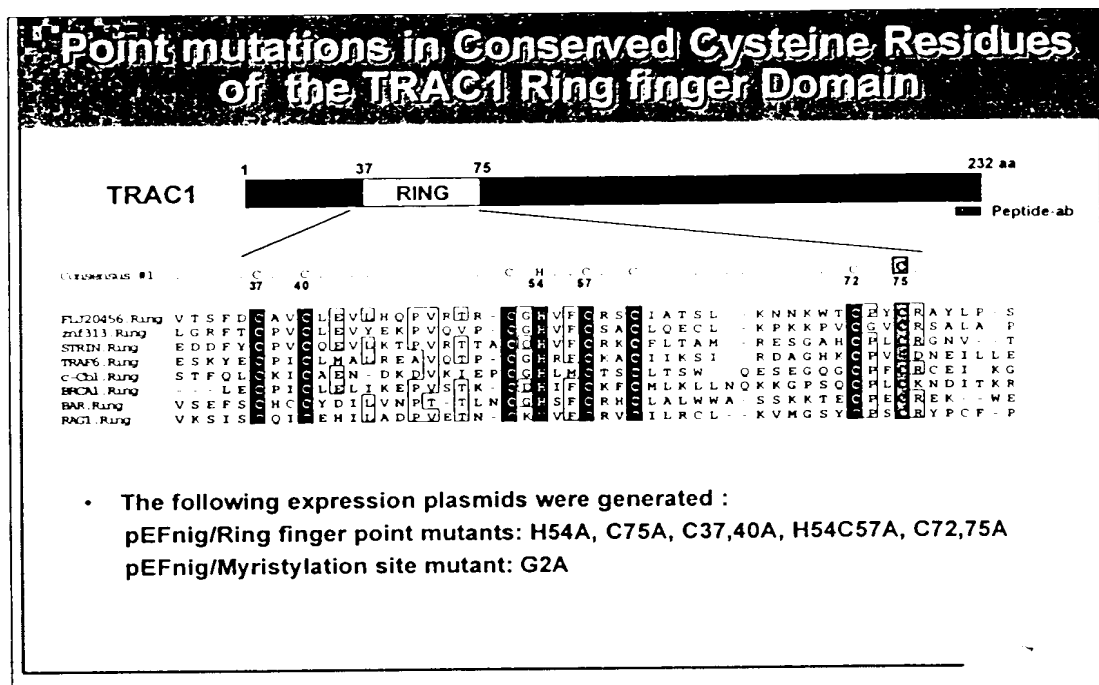


Figure 14

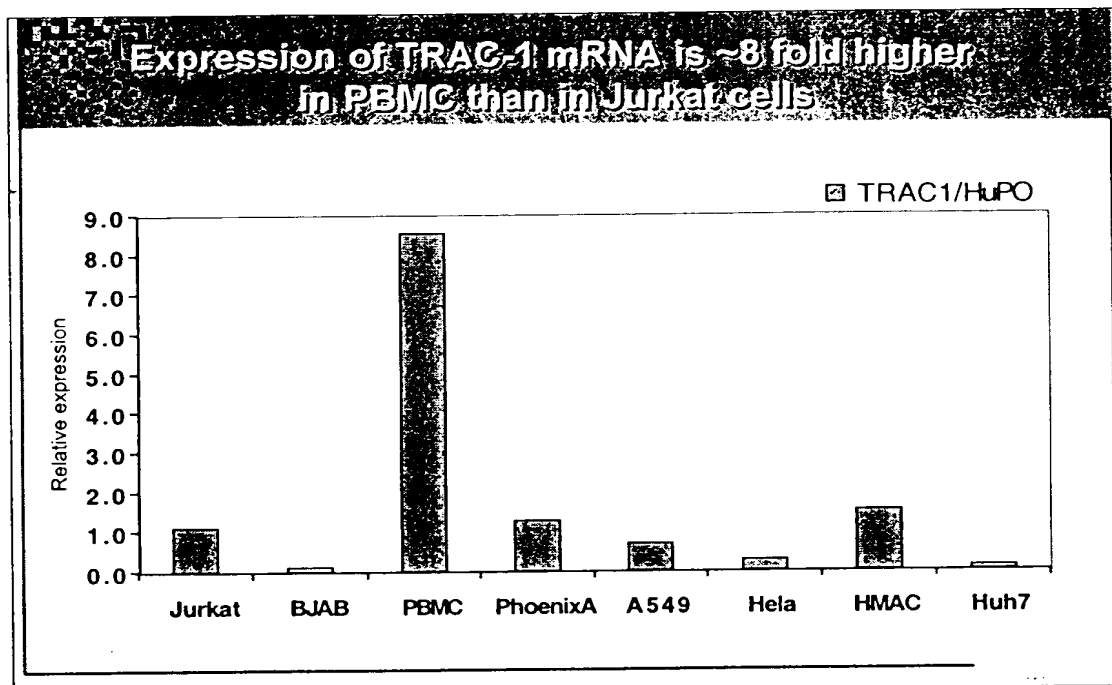


Figure 15

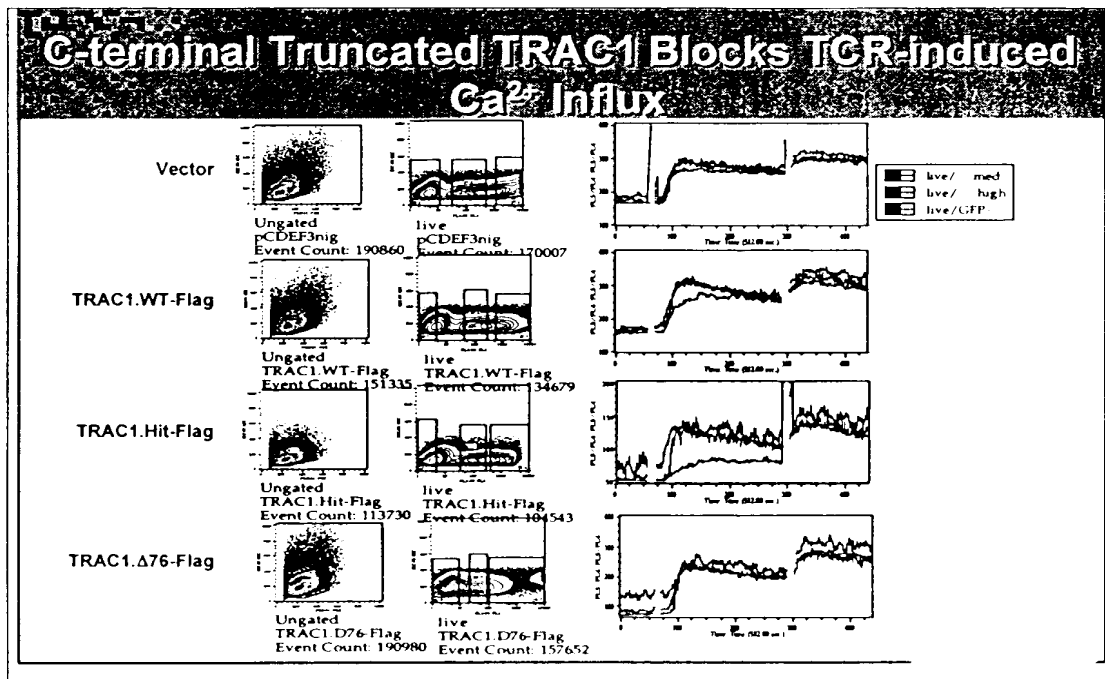


Figure 6

An Intact TRAC1 Ring domain is Required for Inhibition of α -TCR-Induced CD69 Up-regulation

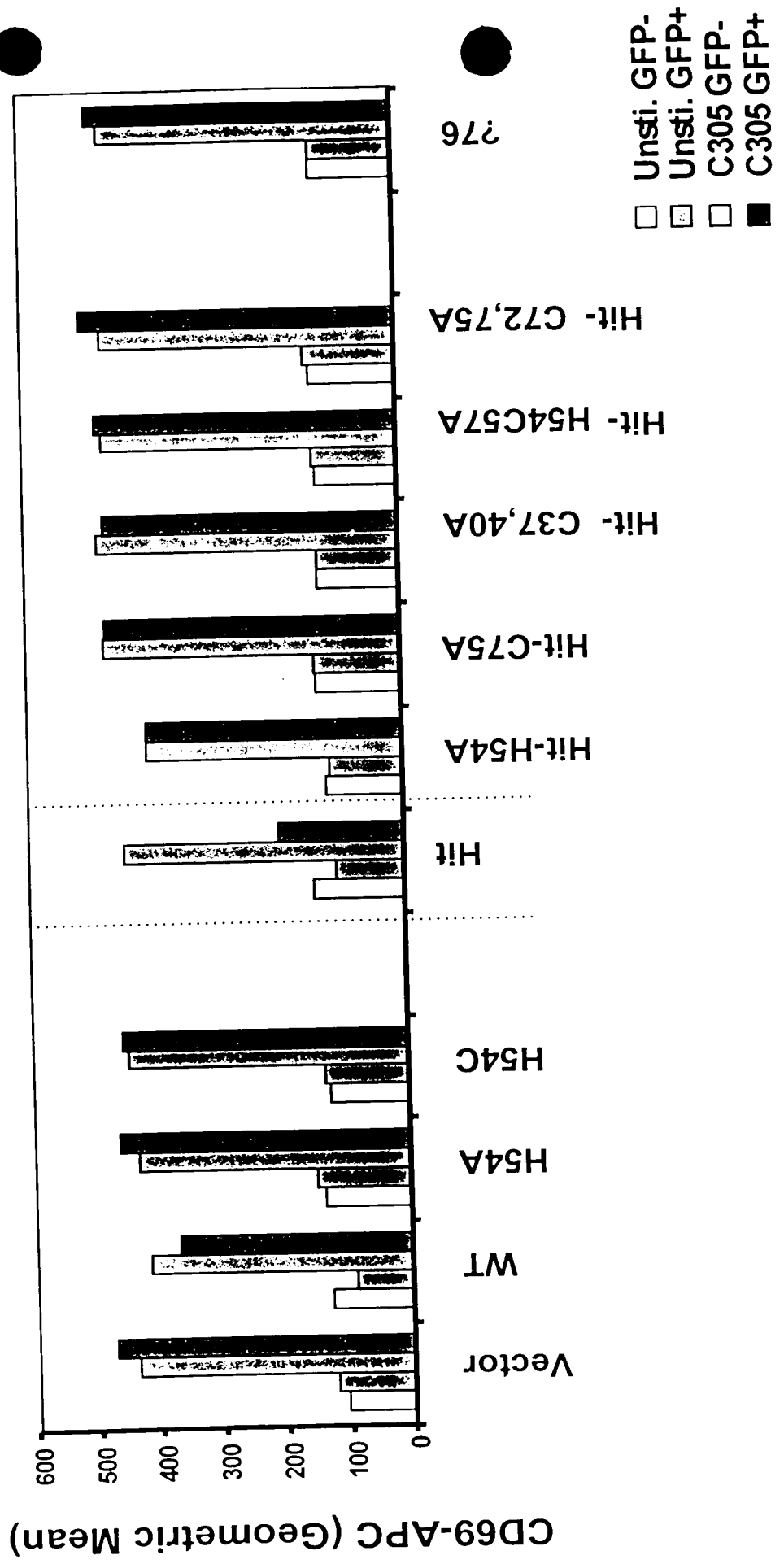


Figure 17

Summary of Functional Effects by Different TRAC-1 constructs

| | | Ubiquitin ligase activity | CD69 induction | Calcium mobilization |
|---------|-----------------------------|---------------------------------|-------------------|-------------------------|
| TRAC1 | 1 37 75 232 aa RING Flag | yes | - | +/- |
| Hit | 1 37 75 170 aa RING Flag | yes | ↓ | ↓ |
| Δ76 | 77 232 aa Flag | no | - | - |
| C37,40A | XING Flag | - | - | - |
| C72,75A | RINX Flag | - | - | - |
| H54C57A | RXING Flag | - | - | - |

Figure 18

Transiently Transfected TRAC1 Protein Binds to Ubiquitin - Conjugating Enzymes (E2s) Ubch7 and Ubch5 *in vitro*

Cellular Lysate
Transfected w/

pEF vector pEF.TRAC1.WT

10% Lysate 10% Lysate

Ni-Ubch7 Ni-Ubch7

Ni-Ubch5 Ni-Ubch5

Ni-beads Ni-beads

Purified E2s
W/ His-tag

kDa

38 —
28 —
17 —
14 —
6 —

← FLAG-TRAC1

Figure 19

